

Reconstruction of Cellular Signal Transduction Networks Using Perturbation Assays and Linear Programming

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Abstract

Perturbation experiments for example using RNA interference (RNAi) offer an attractive way to elucidate gene function in a high throughput fashion. The placement of hit genes in their functional context and the inference of underlying networks from such data, however, are challenging tasks. One of the problems in network inference is the exponential number of possible network topologies for a given number of genes. Here, we introduce a novel mathematical approach to address this question. We formulate network inference as a linear optimization problem, which can be solved efficiently even for large-scale systems. We use simulated data to evaluate our approach, and show improved performance in particular on larger networks over state-of-the-art methods. We achieve increased sensitivity and specificity, as well as a significant reduction in computing time. Furthermore, we show superior performance on noisy data. We then apply our approach to study the intracellular signaling of human primary nave CD4⁺ T-cells, as well as ErbB signaling in trastuzumab resistant breast cancer cells. In both cases, our approach recovers known interactions and points to additional relevant processes. In ErbB signaling, our results predict an important role of negative and positive feedback in controlling the cell cycle progression.

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Introduction

Functional knockdowns for example by RNA interference (RNAi) are a powerful tool to identify genes involved in a specific biological process. The technology has been widely employed in large scale screening approaches, for example to identify genes relevant for cellular growth and viability, for cell proliferation, in bacterial or viral infection, in signaling, in cellular trafficking, influencing the chemosensitivity of tumors, or determining stem cell identity [1–13]. While functional knockdowns are very successful to identify genes associated with a particular phenotype, the spatial and temporal placement of hits in their surrounding signaling or regulatory networks poses considerable challenges [14]. In silico network reconstruction using machine learning methods has been used to infer underlying molecular networks from perturbation data with some success. Approaches suggested include Bayesian [15] or dynamic Bayesian networks [16], probabilistic Boolean threshold networks [17,18], conditional correlation analysis [19], differential equation models [20] and others. For knockdown data with high-dimensional phenotypes acquired for example using microarrays, Nested Effects Models (NEMs) can be used [21–25]. NEMs use the nested structure of phenotypic effects after different knockdowns to infer a hierarchy of genes. The underlying assumption is that if gene A is upstream of gene B in a signaling pathway, then the effects seen after a knockdown of A must be a superset of the effects seen after

knockdown of B. While NEMs were recently extended to handle time-course measurements [26,27], they still have severe limitations when applied to large networks, and they cannot handle combinatorial knockdowns. This, however may be crucial to distinguish between complex network topologies, for example with feed-forward loops. In addition, NEMs require high-dimensional “effects” observations after every knockdown, which are not routinely measured in many perturbation screens. Such phenotypic data furthermore offers only very indirect information about the signaling pathway at hand. Direct observations of protein states cannot be used with NEMs. These limitations were the motivation for the development of Deterministic Effects Propagation Networks (DEPNs) [28]. DEPNs assume deterministic signaling in the underlying network, and introduce noise only at the measurement stage. The measurement distribution of active versus inactive proteins is then estimated from the data either using maximum likelihood inference or maximization of the posterior distribution. Given the measurement distribution and knockdown data, alternative network topologies can then be scored. Dynamic DEPNs (D-DEPNs) have recently been proposed as an extension of DEPNs that explicitly take time course data into account [29].

Besides statistical approaches, also combinatorial optimization methods have been suggested to tackle the problem of inferring a signaling network from perturbation data. Ourfali *et al.* proposed an integer programming approach to infer an integrated protein-protein

and protein-DNA interaction network [30]. The authors used gene expression measurements after knockout experiments combined with database information to reconstruct regulatory pathways in yeast. A similar approach has been suggested by Lan *et al.*, linking genetic and transcriptomic screening data with data of known molecular interactions [31]. The drawback of both approaches is that they need a network to start with, hence, these methods cannot be used if no such prior information is available. Furthermore, Hashemikhabir *et al.* recently showed that even when assuming that an approximately correct network is given, finding the minimum number of topological changes to make this network consistent with given experimental data is an NP complete problem [32]. In fact, the exponentially increasing number of possible networks for increasing number of genes is the most important limiting factor when inferring network topologies. For a directed graph over $n \in \mathbb{N}$ nodes, there are $2^{n(n-1)}$ possible network topologies (forbidding cycles of length one). Complete enumeration of the solution space thus quickly becomes infeasible already for $n > 6$ or 7 [17,21].

In this manuscript, we consider signal transduction as an information flow through a network, that is perturbed by experimental interventions. The idea is to formulate the network inference problem as an integer linear program (ILP), where the $\{-1, 0, 1\}^{n^2}$ solution vector specifies for each ordered pair of nodes if they are linked by an inhibition, by no interaction, or by an activation. However, ILP is an NP hard problem. We therefore drop the integrality constraint, converting the ILP into a non-integral linear program (LP). Edges then have continuous edge-weights, and the decision whether or not to include an edge into the final network requires a heuristic decision using a threshold-based discretization. This formulation of the network inference problem as a LP allows the use of polynomial time solvers such as the ellipsoid method [33]. We here use the simplex algorithm as LP solver. Albeit this algorithm is not necessarily polynomial, it has proven itself to be very efficient in practice [34].

We have implemented this approach in the R programming language [35]. To demonstrate its application, we evaluate our approach on simulated data, and show that it can robustly deal with noisy and missing data. An evaluation on large scale networks shows an over 10 fold decrease in running time whilst demonstrating superior performance over other current state-of-the-art methods. Using the method on signal transduction downstream of CD3, CD28 and LFA-1 in CD4⁺ T-cells, we demonstrate the applicability to real experimental data. Last but not least, we applied the approach to reconstruct ErbB signaling in breast cancer cells. Our approach could successfully reconstruct known interactions, and furthermore pointed to an important role of feedback loops in regulating the cell cycle progression mediated by the ErbB pathway.

Methods

Let an (unknown) graph $\mathcal{G} = (\mathcal{V}, \mathcal{E})$ with nodes $v_1, \dots, v_n \in \mathcal{V}$ and directed edges $e_{ij} \in \mathcal{E}$, $i, j \in \{1, \dots, n\}$ be given. We define the edge e_{ij} to go from node v_i to node v_j . The set of vertices \mathcal{V} corresponds to proteins or protein complexes, and the set of edges describes activatory or inhibitory interactions between proteins, for example by phosphorylation or dephosphorylation. Each protein $v_i \in \mathcal{V}$ is associated with an activity level $x_i \in \mathbb{R}_0^+$, and can either be active ($x_i \geq \delta_i$) or inactive ($x_i < \delta_i$). The parameter $\delta_i \in \mathbb{R}^+$ is a positive, node-specific threshold level. Finally, edges $e_{ij} \in \mathcal{E}$ have weights $w_{ij} \in \mathbb{R}$ associated with them. Activating edges are characterized by $w_{ij} > 0$, inhibiting edge by $w_{ij} < 0$. For notational convenience, we write $w_{ij} = 0$ if $e_{ij} \notin \mathcal{E}$. We now make the assumption that

$$x_i = w_i^0 + \sum_{j \neq i} w_{j,i} x_j, \quad (1)$$

hence the activity level of a protein x_i is fully determined by other proteins x_j in the network with $w_{j,i} \neq 0$. Here, $w_i^0 \in \mathbb{R}_0^+$ is a bias term that describes the baseline activity of x_i in the absence of any external regulations. We then write

$$v_i = \begin{cases} 1 & \text{if } x_i \geq \delta_i \\ 0 & \text{otherwise.} \end{cases} \quad (2)$$

The graph \mathcal{G} is fixed in our setting, but unknown. We can now experimentally perturb \mathcal{G} by forcing individual nodes v_i to the inactive state $v_i = x_i = 0$, and then observe the influence this has on all other nodes in $\mathcal{V} \setminus v_i$.

Our mathematical model of signal transduction is based on the notion of an information flow through \mathcal{G} . The flow begins at one or several *source nodes* $S \in \mathcal{V}$ and is then propagated via the edges \mathcal{E} through the network until it reaches one or several *sink nodes* $F \in \mathcal{V}$. Thus, a protein $v_a \in \mathcal{V}$ influences another protein $v_b \in \mathcal{V}$ if there exists a directed path from v_a to v_b . If there is a direct connection $e_{a,b} \in \mathcal{E}$ we say that v_a is the parent node of v_b and v_b is the child node. According to equation (2), a knockdown of a node v_a implies that its children may change their activity states. The problem we have to solve is to infer the underlying edge weights w_{ij} from observations of node activity levels after a set of such perturbation experiments. The only constraint we impose on \mathcal{G} is that $\forall i : e_{i,i} \notin \mathcal{E}$. Cycles of length > 1 are explicitly permitted.

Now let a set \mathcal{K} of $K \in \mathbb{N}$ different perturbation experiments be given, where each perturbation experiment $k \in \mathcal{K}$ consists of the simultaneous knockdown of one or several nodes in \mathcal{V} . Given experimental data, we define the *observation matrix* $X = (x_{i,k}) \in \mathbb{R}^{n \times K}$, where $x_{i,k} \geq 0$ is the observed activity level of v_i after perturbation experiment k . We here use continuous values for $x_{i,k}$, accounting for diverse types of experimental measurements that quantify protein abundance, for example fluorescence measured after antibody staining. We furthermore define the *activation matrix* $B \in \{0, 1\}^{n \times K}$ as

$$b_{ik} = \begin{cases} 0 & \text{if gene } i \text{ has been knocked down in experiment } k \\ 1 & \text{otherwise.} \end{cases}$$

The matrix B specifies which of the genes in the network were targeted by which knockdown experiment. The respective genes are fixed to the “inactive” state and are no longer subject to regulation by other genes. Since we permit combinatorial knockdowns of multiple genes simultaneously in one experiment, B is a $n \times k$ matrix, and not simply an index vector.

Linear Programming Model

We can now formulate the network inference problem as a linear program. Assuming that biological networks are sparse [36], we minimize the sum of the absolute edge weights $\sum_{i,j} |w_{ij}|$ and bias terms $\sum_i w_i^0$. We furthermore introduce slack variables $\xi_l \in \mathbb{R}_0^+$, $l = 1, \dots, L$ that permit slight violations of constraints of the LP model and can thus account for noise in the experimental data. To minimize the extent to which slack variables are used, we

include the sum of the slack variables in the objective function. The variable L describes the cardinality of the set of inactive genes in the experimental data, $L = |\{x_{ik} | x_{ik} < \delta_i, \forall i, k\}|$, and corresponds to the number of constraints that may be violated in the linear program. The full LP then becomes:

$$\min_{w_{ij}, w_i^0, \xi_l} \sum_{i,j} |w_{ij}| + \sum_i w_i^0 + \frac{1}{\lambda} \sum_l \xi_l \quad (3)$$

subject to the constraints

$$\forall (i,k) \text{ where } x_{i,k} \geq \delta_i \text{ and } b_{i,k} = 1 : w_i^0 + \sum_{j \neq i} w_{j,i} x_{j,k} \geq \delta_i \quad (4)$$

$$\forall (i,k) \text{ where } x_{i,k} < \delta_i \text{ and } b_{i,k} = 1 : w_i^0 + \sum_{j \neq i} w_{j,i} x_{j,k} \leq 0 + \xi_l \quad (5)$$

The constraints (4) and (5) are defined for each pair (i,k) and specify the effect of the knockdown k on gene i . According to equations (1) and (2), for given knockdown k , the activity of each gene i is determined by the activities of its parents $x_{j,k}$, the strength of their influence $w_{j,i}$, and gene i 's baseline activity w_i^0 . Thus, if gene i is active after perturbation k , that is, if $x_{i,k} \geq \delta_i$, and gene i has not been silenced in knockdown k ($b_{i,k} = 1$), constraint (4) has to hold. Similarly, if the gene is inactive, we require $v_i = 0$, and hence constraint (5) has to hold. We note that we do not need to consider observations $x_{i,k}$ for the (i,k) pairs where $b_{i,k} = 0$, since these correspond to perturbed genes directly targeted by the knockdowns. The respective genes/proteins are thus no longer influenced by incoming regulations. Furthermore, the constraints (4) and (5) relax equation (2) in that equality is no longer required, but instead a margin of δ_i is enforced between activated and non-activated node states.

Missing observations $x_{j,k}$ can heuristically be treated in this framework as follows: If in constraints (4) or (5), a variable $x_{i,k}$ is missing on the left hand side, the constraint is simply left out. If one of the $x_{j,k}$ is missing on the right hand side, the corresponding worst-case is assumed, i.e. in case of constraint (4), the missing value is assumed 0, whereas in constraint (5), the missing value is assumed to be 1.

The function of the slack variables is to allow violations of the constraints (5), in case of contradictions between constraints (4) and (5). The parameter $\lambda \in \mathbb{R}_0^+$ is a non-negative penalty parameter to control the introduction of slack variables ξ_l in constraint (5). Intuitively, if $\lambda = \infty$, the slack variables can become infinitely large without affecting the objective function (3); conversely, if $\lambda = 0$, slack variables are not allowed. We use leave-one-out cross-validation (LOOCV) to choose λ optimally for a given data set. To restrict the introduction of slack variables, we restrict λ to be at most $L * \sigma^2(x_{i,k})$, where $\sigma^2(x_{i,k})$ is the variance of the observations $x_{i,k}$ for all (i,k) . Thus, the higher the variance of the data the higher the slack variables can become. The upper bound is chosen based on the worst case where all L slack variables are unequal to zero.

Inclusion of Prior Knowledge

In many cases, some knowledge about the biological processes underlying a particular data set will already be given. This can be used to formulate additional constraints, for example requiring

certain edge weights $w_{i,j}$ to be above or below a certain threshold if it is known that the respective proteins do or do not interact. Similarly, if it is known which proteins v_i are receptors (source nodes S) or sink nodes F , the following additional constraints can be included:

$$\forall i \text{ where } i \in V \setminus S : \sum_{j \in V, j \neq i} w_{j,i} \geq \delta_i \quad (6)$$

$$\forall i \text{ where } i \in V \setminus F : \sum_{j \in V, j \neq i} w_{i,j} \geq \delta_i \quad (7)$$

The constraints force each node that is not a source or sink node to have at least one incoming and one outgoing edge.

Data Simulation and Network Inference on Simulated Data

To evaluate our model on simulated data, we used network topologies that were taken from the KEGG database [37] as ground truth. We randomly extracted sub-networks from randomly selected KEGG signaling networks, for details see file S1. Only gene-gene interactions in KEGG were considered. We then simulated single knockdowns of every protein i in each of the networks, double knockdowns of $n/2$ randomly chosen protein pairs, as well as one experiment without any perturbation. Data simulation was done using equations (1) and (2), by setting $w_{i,j} = 1$ for all edges $e_{i,j} \in \mathcal{E}$. Nodes v_i without incoming edges were assumed to have $x_{i,\cdot}$ sampled from a Normal distribution with mean 0.95 and standard deviation σ , unless v_i was directly targeted by the knockdown. To then simulate measurement data from the simulated node activities, we employed two Gaussian probability distributions, one for active and one for inactive proteins. Continuous observations of an activated node were simulated from the normal distribution $\mathcal{N}(0.95, \sigma)$, or from $\mathcal{N}(0.56, \sigma)$ for inactive nodes, in line with the procedure employed by Fröhlich *et al.* [28]. The values of the means in the two Gaussians were chosen to agree with average levels of activatory and inhibitory proteins as we observed in the ErbB data set [28]. The parameter σ was chosen as described below, δ_i was generated with a normal distribution of $\mathcal{N}(0.755, \sigma)$. We generated data with three replicates for each type of experiment. For the network inference with the LP model, the replicates have been summarized using the arithmetic average.

To find the best parameter λ in the range $0 \leq \lambda \leq L * \sigma^2(x_{i,k})$ and to compute a range of possible weights for each edge we used LOOCV with a grid search. The basic idea is to leave out parts of the observational data, infer networks on the remaining data with different values of λ , and use the resulting networks to predict activity levels of data that were left out in network inference. This prediction was repeated 100 times, and we calculated every time the MSE between the predicted and observed activity levels. The best parameter λ is the one with minimal MSE. For evaluation of resulting networks, we computed the median and the median absolute deviations (MAD) of the edge weights learned in each step. This is necessary, since different weights can be learned for individual interactions in each cross-validation step. We included only robustly learned edges in the final network, requiring that the median of the learned edges from the different crossvalidation runs

was larger than the median absolute deviation (MAD) over the runs.

We simulated data based on ten-node networks to evaluate the performance of our approach on noisy and missing data. We furthermore tested how the introduction of prior knowledge improves results. Furthermore, we applied our approach on simulated data of larger networks, to assess performance on bigger problems and to measure how the computational time increases with increasing network size.

Ten-Node Networks

We extracted ten different networks from KEGG with $n = 10$ nodes each. The extracted networks have a varying number of edges: five networks have seven interactions, the remaining networks have five, eight, ten, twelve and thirteen interactions, respectively. All edges were assumed positive, i.e. there are no inhibitions in the simulated networks. To assess how our inference approach performs on noisy data, we simulated different noise levels in the generated data by varying σ with values of 0.01, 0.05, 0.14 and 0.2. For the evaluation of our model on incomplete data, we randomly selected 10%, 20%, 40% and 50% of the genes and removed all the measurements given for them. Thus, 10% missing values corresponds to one gene without any observations. We repeated data simulation in this way 10 times for each network and each percentage. For the simulation of missing data for the ten-node networks, a noise level of $\sigma = 0.01$ was employed in the two Gaussian distributions describing measurement noise.

Lastly, we tested how the integration of prior knowledge improves network inference. We therefore randomly selected 10%, 25%, 50% and 100% of the true interactions, and included the additional constraints $w_{ij} > 1$ for these edges in the inference. In addition, we separately inferred the networks assuming that the identities of the source and sink nodes are given, but assuming no knowledge about edges, again using $\sigma = 0.01$.

Larger Networks

To evaluate the performance of our LP model on larger problems, we extracted five networks from KEGG with $n = 16, 26, 28, 44$ and 52 nodes, respectively. As above, we simulated only activatory interactions, with 17, 27, 31, 43 and 51 edges in the five networks. We then simulated single knockdowns of every node, $n/2$ randomly chosen double knockdowns, and one experiment without any knockdown for each of the networks, and reconstructed the underlying networks from the simulated data alone using our LP approach. In contrast to inference on the ten-node networks, we switched from LOOCV to ten-fold crossvalidation for the estimation of λ . This significantly reduces the number of times the training process is repeated and thus the total run time.

Evaluation of Inference Results

For both, the ten-node networks and the large-scale problems, we compared our results with those derived with the recently published DEPN approach [28]. For each generated data set, we inferred network topologies using the LP model and the DEPN approach, calculated receiver operating characteristic (ROC) [38,39] curves of the learned interactions and computed the area under the curve for the ROC-curve (AU-ROC) and the precision-to-recall curve (AU-PR). To assign a weight to each edge for the DEPN approach, we used greedy hillclimbing and bootstrapping (resampling with replacement) with 100 bootstrap samples, as proposed in the DEPN implementation [28]. We furthermore considered only edges appearing with a frequency higher than 0.5 for the evaluation. Since the DEPN approach cannot infer

negative interactions, we treated our LP model similarly and ignored the signs of the edge weights.

We note here that there are two different philosophies underlying the networks reconstructed by the DEPN approach and by our method. In the DEPN approach, an edge $v_a \rightarrow v_b$ implies that v_b is downstream of v_a in the network, and will be affected by a knockdown of v_a . Therefore, DEPNs assume transitivity: If there are edges $v_a \rightarrow v_b$ and $v_b \rightarrow v_c$, then the DEPNs also infer an edge $v_a \rightarrow v_c$, since a knockdown of node v_a will affect node v_c indirectly via v_b . DEPNs thus return equivalence classes of networks, and not a single unique network. Our interpretation is different: We interpret edges as *direct* physical interactions between molecules, and lack of an edge means that there is no direct interaction between the molecules. Edges are then not transitive. This leads to a fundamental difference to the DEPNs: Provided sufficient data are available, a unique minimal network can be inferred from the data. We believe that, in a biological setting, one is usually interested in inferring the actual network of physical interactions, and not a transitively closed network of upstream-downstream relations. We therefore in the following show the performance evaluation based on the actually inferred networks (i.e. we compare the single network inferred by our approach and the transitively closed network returned by the DEPN against the gold standard network). This comparison is biased, since a whole equivalence class is compared against a single network for the DEPN approach. As an alternative, we compared the DEPN results against the transitive closure of the reference network, thus comparing the two equivalence classes; these results are given in figure S1 and table S1.

Network Inference on Real Data

Simulated data can be used to study the effect of different characteristics of data on network inference performance, however, only an evaluation on real data can provide a realistic picture of the practical applicability of a method. To assess performance of our approach on real world problems, we used two different publicly available data sets: The first data set focuses on the signal transduction downstream of CD3, CD28 and LFA-1 in primary nave CD4⁺ T-cells [15], the second data set considers ErbB signaling in a breast cancer cell line [28]. We compared performance of our approach on both data sets with random guessing and inference using the DEPN approach, and with results of the Bayesian approach employed by Sachs *et al.* in case of the CD4⁺ T-cell data [15].

CD4⁺ T-Cell Signaling after CD3, CD28 and LFA-1 Stimulation

The first data set we used regards an intracellular signaling network in human primary nave CD4⁺ T-cells. This data set was published by Sachs *et al.* in 2005, and comprises nine perturbation experiments (overactivations and inhibitions) with effects quantified using flow cytometry [15]. Given are measurements of the 11 phosphorylated proteins and phospholipids PKC, PKA, Akt, Raf, Mek1/2, Erk1/2, p38, JNK, PIP2, PIP3, PLXγ downstream of CD3, CD28 and LFA-1. The perturbation conditions consist of four stimulatory experiments and five inhibitions. Quantitative single cell measurements are given for each of the 11 phosphorylated proteins in each perturbation condition. We normalized the fluorescence signals of the single-cell flow cytometry data against the cell size and against overlapping wavelength ranges of the emission signals of the fluorophores used for the 11 molecules, as described in file S1. We then sampled from the data using bootstrapping to get 10 bootstrap samples with three replicates

each from the data. The replicates were then further summarized by taking the median. We inferred a network for each bootstrap sample using the DEPN and LP approaches. Inference with the LP model was carried out with parameters δ_i set to the median of the nine measured conditions for each molecule i . LOOCV was used to determine λ , where parameters of the two normal distributions for active and inactive states were determined from measurements of the activated respectively the inactivated molecules. These distributions were then used in the cross-validation runs to predict left-out protein states required for the MSE computation, and optimal λ were then used for final network predictions. Edge weights from the LOOCV were summarized across the bootstrap samples using the median. Additional details are given in file S1. Inference using the DEPN approach was performed using greedy hillclimbing and bootstrapping with 100 bootstrap samples for each of the sampled data sets. The median of the inferred edge weights over the samples was used for final evaluation.

ErbB Signaling in Breast Cancer Cells

As a second evaluation on real data, we used recently published data on ErbB signaling in a breast cancer cell line. The ErbB signaling pathways are some of the best studied signaling networks and it is known that they regulate diverse physiological responses such as cell division, motility and survival [40]. Fröhlich *et al.* focused on the 16 proteins ERBB1, ERBB2, ERBB3, IGF1R, ER-alpha, pAKT1, pERK1/2, MYC, Cyclin D1, p27, p21, Cyclin E1, CDK6, CDK4, CDK2 and pRB1. The proteins are all involved in the ErbB receptor-regulated G1/S cell cycle transition network. For a detailed description of the experimental setup see [28]. In short, the authors used RNAi knockdowns followed by reverse phase protein arrays (RPPA) [41,42] to quantify protein levels. They performed single-knockdowns of the thirteen proteins ERBB1, IGF1R, ER-alpha, pAKT1, pERK1/2, MYC, Cyclin D1, p27, p21, Cyclin E1, CDK6, CDK4, CDK2 and three double-knockdowns of ERBB1+ERBB2, ERBB2+ERBB3 and ERBB1+ERBB3 with chemically synthesized siRNAs as well as one experiment with mock transfected cells as a negative control. RPPA measurements were done before and twelve hours after EGF stimulation for ten intermediates of the network, namely ERBB1, ERBB2, pAKT1, pERK1/2, Cyclin D1, p27, p21, CDK4, CDK2, pRB1, to quantify their protein expression after each individual perturbation. This was repeated in four technical and three biological replicates, which were normalized by the authors using quantile normalization. The remaining proteins could not be quantified due to lack of antibodies suitable for RPPA.

We preprocessed this data further by summarizing replicate measurements using the arithmetic mean. We then solved the LP model based on the data measured 12 hours after the EGF stimulation, using δ_i set to the average of the mock control at time zero for the respective protein. We used the constraints (6) with source nodes ERBB1, ERBB2 and ERBB3, and (7) with sink node pRB1. LOOCV was used to estimate $\lambda = 1.83$.

Results

We implemented the linear programs in the statistical programming environment R version 2.12.1 [35]. The R cran package “lpSolve” version 5.6.5 was used to solve the linear programs. This package implements the simplex LP solver. The R package “network” was used for graph handling. All calculations were performed on a 3 GHz Intel dual-processor Xeon quadcore computer with 32 GB RAM, running the Linux operating system.

No parallelization was used in the computations. Data were simulated as described in methods and analyzed using the network inference approach developed. We studied the effect of different levels of noise and missing values on inference performance on simulated data, as well as effects of overall network size. Results of our linear programming approach were compared with DEPNs as well as random guessing, showing superior performance of our approach. We then applied our method to reconstruct signaling downstream of CD3, CD28 and LFA-1 in CD4⁺ T-cells, as well as signal transduction in the ErbB pathway in breast cancer cells.

Analysis on Simulated Data

Simulated data allows a systematic evaluation of network inference performance under well defined conditions. The “gold standard” network used to simulate data is known, hence network inference results can directly be evaluated. Furthermore, full control over properties of the data can be exerted, and it is thus possible to systematically study the influence of different levels of noise, missing values, or network size on inference performance. We performed network reconstruction under differing conditions on simulated data, and evaluated results using receiver operator characteristic (ROC) curves and precision-recall (PR) analysis. As a single measurement of inference performance, the area under the ROC and PR curves was used. For all analyses on simulated data, we assumed no further prior knowledge about the underlying network, in particular, we did not specify which of the nodes were source or sink nodes in the network. Constraints (6) and (7) were hence not used.

Ten-Node Networks

As a first evaluation of our approach, we reconstructed networks from the simulated ten-node data sets, without noise or missing values. Reconstruction was done on averages of three simulated replicates for each of the ten networks, and the area under the ROC and PR curves was calculated for each of the 10 reconstructions. Figures 1A and 1B show the distribution of the AUC ROC and AUC PR values so obtained, respectively, over the 10 simulated networks. We furthermore used the DEPN approach on the same data, and provide its performance for reference in the figures. Furthermore, the rightmost boxplot in the figures shows the achieved performance for random guessing, derived by 100 fold random permutation of edges in the true network – thus guessing a network with the same number of edges as the true network.

We observed superior performance of the LP approach both in terms of the AUC ROC and AUC PR evaluation. This result is somewhat surprising, since the method used for data simulation is closely related to the model assumptions made by the DEPN approach, with a deterministic signal transduction and noise introduced only at the level of the experimental measurements. Variability of performance is comparable across the two methods, with interquartile ranges of approximately 0.3 for the AUC ROC and almost 0.6 for the AUC PR. Both approaches perform significantly better than random guessing, indicating that both methods are able to extract information about the underlying signal transduction networks from the knockdown data. We note that the small values for the AUC PR are due to the fact that the selected sub-networks are all sparse, a property they inherit from the networks stored in the KEGG database.

We next assessed performance of our approach under increasing levels of noise in the experimental data. This was achieved by increasing the variability of the normal distributions used to simulate measurements, as described in methods, using standard deviations σ of 0.01, 0.05, 0.1 and 0.2, and again

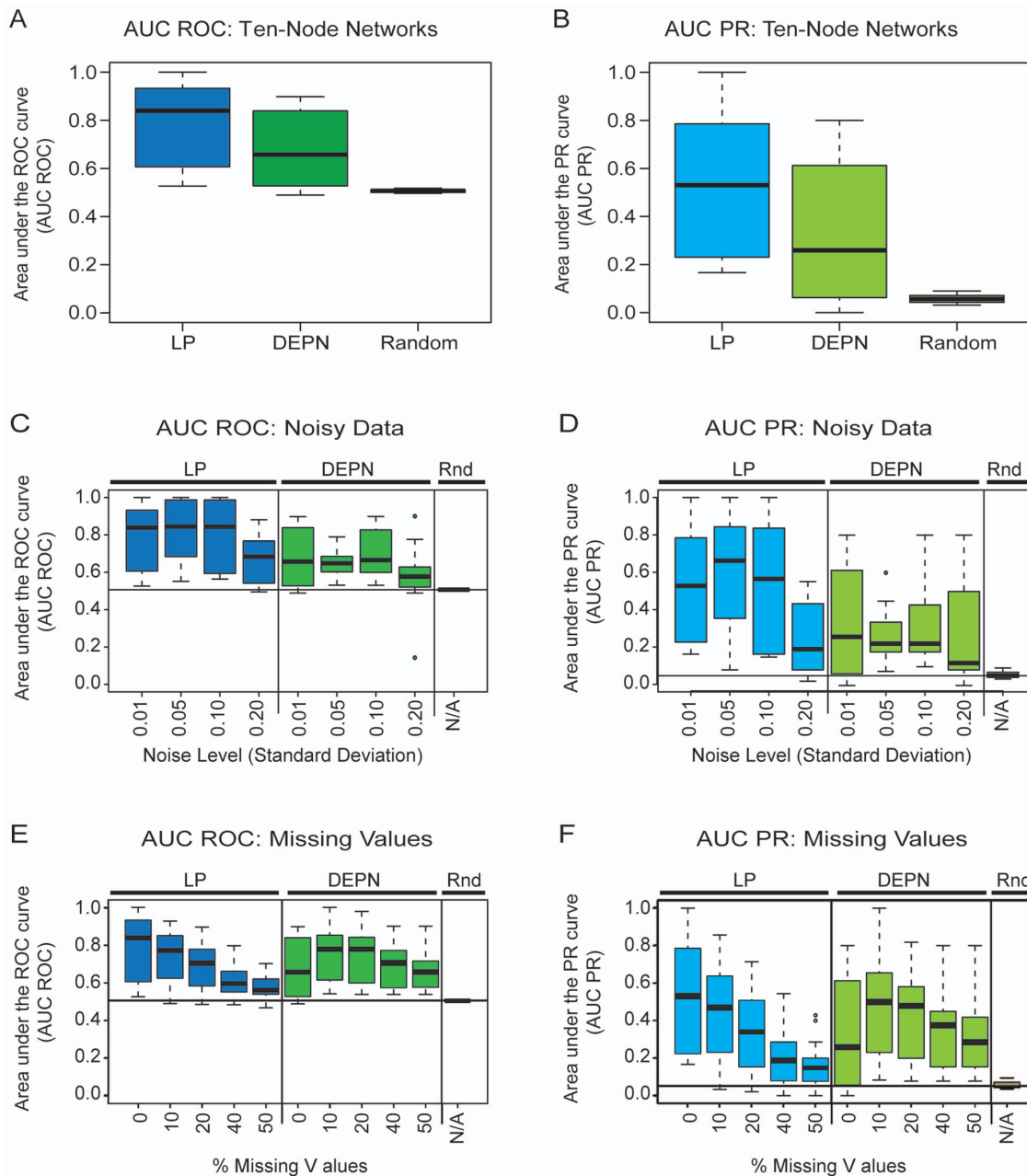


Figure 1. Evaluation on simulated data for small-scale network reconstruction. The figure shows the area under the receiver operator characteristic (AUC ROC) and area under the precision-recall (AUC PR) curves on simulated networks of size ten nodes. Shown are crossvalidation results over 10 simulated data sets, with reconstruction performed using the Linear Program (LP), Deterministic Effects Propagation Networks (DEPN) and random guessing (Rnd). (A) and (B) show performance on data with low noise ($\sigma=0.01$) and with no missing values, (C) and (D) illustrate performance effects of increasing levels of noise, and (E) and (F) regard effects of missing values on inference results.
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summarizing data from three replicates and assessing performance over ten different networks. Results are shown in figures 1C and D, showing the distribution of achieved AUC ROC and AUC PR values for the LP model, DEPN, and random guessing (Rnd). Guessing is done independently of the actual data, and hence uninfluenced by the level of noise in the data. As expected, performance of both network inference approaches deteriorates with increasing levels of noise, but all approaches remain superior to guessing even for the highest level of noise simulated. We consistently observed better performance of the LP approach, indicating that our method can adequately handle noisy data.

As a further performance test, we evaluated the effect of missing data on reconstruction performance. We left out up to 50% of the data, and reconstructed networks using only the remaining values (figures 1E, F). This resulted in a performance decrease for the LP and the DEPN approach, however, both methods are still better than random guessing even when 50% of the data are missing.

Furthermore, we tested the impact of prior knowledge on network reconstruction performance. For this purpose, we either disclosed the identity of source and sink nodes in the true underlying network by using constraints (6) and (7), or we added additional constraints to force 10%, 25%, 50% or 100% of the

true edges in the gold standard network to have weight $w_{ij} \geq 1$, thus requiring the edge to be present in the reconstructed network. As expected, the more prior knowledge we included in the model the better are the resulting predictions, compare figure S2.

To evaluate the performance of our approach on networks having inhibitory interactions, we randomly selected half of the edges of each of the ten-node networks to be deactivating. We simulated data for these networks similarly to the networks having only positive interactions with $\sigma=0.01$. We then applied our inference approach on the data and assessed the performance by computing AUC ROC and AUC PR values (figure S3). In spite of the additional complexity of the three class problem (activation, inhibition or no edge), overall performance is only marginally affected.

Larger Problems and Runtime Analysis

We next assessed performance of our network inference approach on larger networks with 16 to 52 nodes. Data were simulated as described in methods, and network inference was carried out using 10-fold crossvalidation. To summarize results, crossvalidation runs were aggregated by using the median of the crossvalidation runs for each individual edge weight. We compared results with the DEPN approach and with random guessing. Figure 2 shows a comparison of the resulting AUC values from the receiver operator characteristic (2A) and precision-recall analysis (2B). On these larger networks, AUC-ROC values between 0.6 and 0.7 and AUC-PR values around 0.4 were achieved consistently for all network sizes tested using the linear programming approach, whereas the DEPNs were only marginally better than random guessing.

To assess runtime performance of the network inference, we measured the average required time to infer the underlying network for our LP model and the DEPN approach. Figure 3 shows the measured running times for the two approaches on networks of increasing size; note the logarithmic scale of the Y-axis. The LP model requires on average 7.12 ± 4.47 (mean \pm standard deviation) minutes for the ten-node data sets with 16 simulated knockdowns. This is a significant speedup over the DEPN approach on the same network, which requires 24.37 ± 10.01 minutes and even yields inferior reconstruction results (Figure 2). For networks of size $n=44$ and $n=52$, crossvalidation computations with the DEPN approach took over 1000 hours, and were then interrupted. The crossvalidation runs

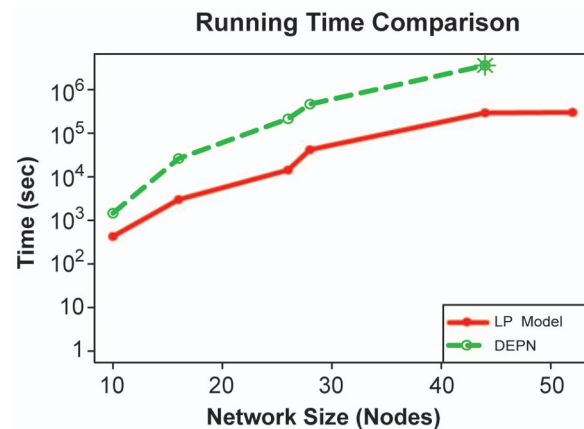


Figure 3. Running time. The figure shows the computation time required to infer networks of different sizes, for the LP (red solid circles and line) and DEPN (green dashed circles and line) approaches, in seconds. Note the logarithmic scale of the y-axis. Computation time is for full evaluation of stratified 10-fold crossvalidation. Computations for networks for 44 and 52 nodes with DEPN were aborted after 1000 hours (green star) without a solution. doi:10.1371/journal.pone.0069220.g003

for the corresponding linear program could still be finished within 85 hours, giving an at least 12 fold decreased runtime.

Evaluation on Flow Cytometry Data

An evaluation on simulated data has the advantage that data properties and simulation conditions can be tightly controlled and a gold standard network for performance evaluation is available. However, only an assessment on real data can ultimately proof practical applicability of an approach in a biological setting. We therefore evaluated network inference performance of our approach on published flow cytometry data, studying 11 phosphorylated proteins and phospholipids downstream of CD3, CD28 and LFA-1 activation in human primary nave CD4⁺ T cells. We compared results obtained using our approach with the network as published by Sachs *et al.* as a reference network [15]. Notably, the Bayesian network approach by Sachs *et al.* exploits individual cell measurements from flow cytometry data, which were summarized to average values for the inference with DEPN and LP model. The amount of data exploited for the inference is

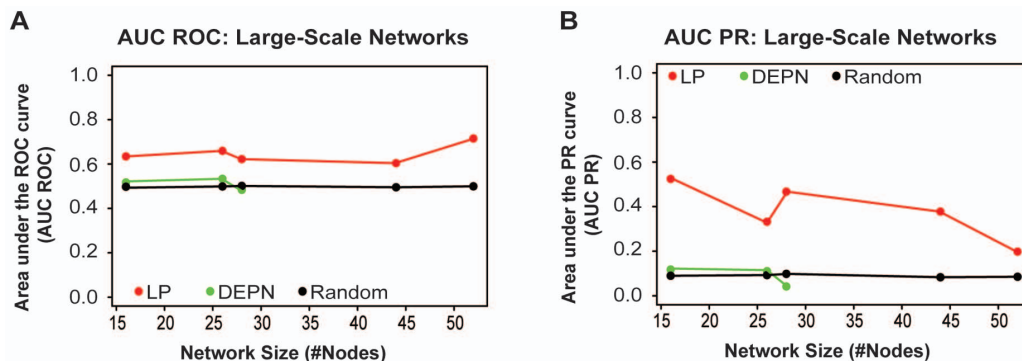


Figure 2. Impact of network size. Effect of network size on network reconstruction – AUC values of the ROC (A) and the PR (B) curves of the network inference using the LP model, the DEPNs and random guessing, for different network sizes. Results were obtained using stratified 10-fold crossvalidation. Calculation with DEPN did not finish within 1000 hours of computation time for networks of size > 30 , and computations were thus interrupted. doi:10.1371/journal.pone.0069220.g002

thus substantially smaller for the latter two approaches. We then calculated true positive edges (TP), false positive edges (FP), true negative edges (TN) and false negative edges (FN) and used these values to compute sensitivity, specificity, precision and accuracy of the network reconstruction. Table 1 summarizes the results for the LP model, DEPN, the Bayesian approach pursued by Sachs *et al.*, and random guessing. P-values were obtained based on the empirical distribution for guessing. The Bayesian network approach pursued by Sachs *et al.* achieved superior results for all of the above measures, with an achieved specificity of 95% ($p < 0.00001$), sensitivity of 71% ($p < 0.00001$), precision of 71% ($p < 0.00001$) and accuracy of 92% ($p < 0.00001$). This is likely due to the substantially larger amount of data available from using individual cell measurements, which had to be summarized for the DEPN and LP models. Performance of both the LP model and the DEPN approach was inferior to the Bayesian network, with specificity 91% ($p < 0.00001$), sensitivity 18% ($p = 0.47$), precision 25% ($p = 0.1$) and accuracy 81% ($p = 0.02$) for the LP model, and specificity 93% ($p < 0.00001$), sensitivity 12% ($p = 0.72$), precision 22% ($p = 0.32$) and accuracy 82% ($p = 0.01$) for the DEPN approach.

Hence, with comparable accuracy, the LP approach achieved higher sensitivity and precision than the DEPN, at the expense of inferior specificity. Notably, if we score only correctly predicted interactions between two proteins, not taking directionality of the interaction into account and consider direct edges that arise from indirect regulations (through intermediate proteins) as correctly scored, the LP model achieves a specificity of 98% ($p < 0.00001$), sensitivity of 53% ($p < 0.00001$), precision of 82% ($p < 0.00001$) and accuracy of 92% ($p < 0.00001$), achieving higher specificity and higher precision also than the Bayesian network. Table 1 furthermore reports results for random guessing, which were obtained using 100-fold random permutation of edges in the reference network.

Reconstruction with our LP approach on the full data set led to the reconstructed network shown in figure 4 (table S2 reports the edge weights). Blue and red edges are true and false positives,

respectively, dashed lines correspond to false negative edges and green lines are edges where a link between the two proteins exists, but the directionality of the interaction was predicted incorrectly. Regarding the inferred edges more closely, we observed that the edges from PIP2 to Erk and from PIP3 to Erk inferred with the LP model are given as indirect connections via $PKC \rightarrow Mek$ and via $PIP2 \rightarrow PKC \rightarrow Mek$ in the reference network. Furthermore, the learned activation of PKC by PKA has already been predicted (albeit in reversed direction) by Sachs *et al.*

ErbB Signaling is controlled by Negative and Positive Feedback Loops

We next applied our approach to publicly available reverse phase protein array measurements obtained after knockdowns and pathway activation in the ErbB pathway [28]. For a description of data and inference procedure, see methods. The inferred network using the LP approach consists of 43 interactions (34 activations and 9 inhibitions). Figure 5 shows a heatmap of the reconstructed edge weights, where the w_{ij} are color coded: pink corresponds to no interaction (zero edge weight), blue to an activation, and yellow to an inhibition. Detailed median edge weights with error bars (median absolute deviation, MAD) are given in table S3. For further analysis, we removed all interactions with coefficient of variation larger than one, thus removing edges with high uncertainty. This procedure resulted in a network with a total of 35 interactions (31 activations, 4 inhibitions), which we analyzed further.

Concerning inhibitory interactions, our inference predicts a strong inhibition of CDK2 by p21, with edge weight -1.07 . This is a known inhibition that has previously been reported [43]. Furthermore, we inferred deactivations of pERK1/2 by pRB1 and CDK2 by Cyclin D1, with edge weights -0.6 and -0.36 , respectively. Both inhibitions seem biologically plausible feedback loops to control the G1/S cell cycle transition, but have not previously been reported. However, there is some evidence showing co-precipitation of CDK2 and Cyclin D1 [44]. Last but not least, we predict an inactivation of p21 by ERBB1 with strength $w_{ij} = -0.2$. This inhibition can be found in the literature as an indirect path via pAKT1 and MYC [28]. Comparing these results with the DEPN approach run on the same data, we firstly have to emphasize that DEPNs cannot infer negative edge weights, and therefore are not able to directly learn any inhibitory influences. Nevertheless, all of the interactions mentioned above with the exception of the pRB1 \rightarrow pERK1/2 inhibition have been inferred using the DEPN approach as unsigned interactions.

Regarding activations, the strongest inferred activation of our LP approach is the activation of pERK1/2 by p21, with $w_{ij} = 1.62$. This result suggests the presence of a strong positive feedback loop controlling the G1/S cell cycle transition. Interestingly, albeit no direct activation of ERKs by p21 has been reported previously, it is known that p21 strongly increases the phosphorylation of cFos and MBP by ERK1 and ERK2 [45], thus constituting a feedback on regulatory effects mediated by ERK. We furthermore predict an activation of CDK2 by ERBB2 with weight $w_{ij} = 1.0$. Although the direct connection of these proteins has not been reported in the literature, there exist two indirect signaling paths: $ERBB2 \rightarrow pAkt1 \rightarrow MYC \rightarrow Cyclin E1 \rightarrow CDK2$ and $ERBB2 \rightarrow pERK1/2 \rightarrow MYC \rightarrow Cyclin E1 \rightarrow CDK2$, which support our results [28]. Our approach furthermore predicts five interactions with edge weights of ≈ 0.95 each: MYC activates CDK6, IGF1R activates Cyclin E1 and vice versa, CDK6 activates ERalpha and ERalpha activates MYC. The last activation is known from literature [46]. The connection between IGF1R and Cyclin E1 is known by an indirect path via pERK1/2

Table 1. Evaluation results on T-Cell signaling.

	LP model	LP model REP	DEPN	Sachs <i>et al.</i>	random
TP	3	9	2	12	2.72
TN	95	102	97	99	91.72
FP	9	2	7	5	13.28
FN	14	8	15	5	13.28
SP	0.91**	0.98**	0.93**	0.95**	0.86
SN	0.18	0.53**	0.12	0.71**	0.16
PR	0.25	0.82**	0.22	0.71**	0.16
AC	0.81*	0.92**	0.82*	0.92**	0.76

The table shows performance measures for the network inference on flow cytometry data regarding signaling downstream of CD3, CD28 and LFA-1 in CD4⁺ T-cells. Network inference was performed using the linear program (LP), Deterministic Effects Propagation Networks (DEPN), random guessing, and a Bayesian network model as implemented by Sachs *et al.* TP = true positives, TN = true negatives, FP = false positives, SP = specificity, SN = sensitivity, PR = precision, AC = accuracy. The column "LP model REP" corresponds to the evaluation results of the LP model where the reversely inferred edges and reported indirect regulations are counted as true positives. Statistically significant differences are marked with ** ($p < 0.0001$) and * ($p < 0.05$), respectively.

doi:10.1371/journal.pone.0069220.t001

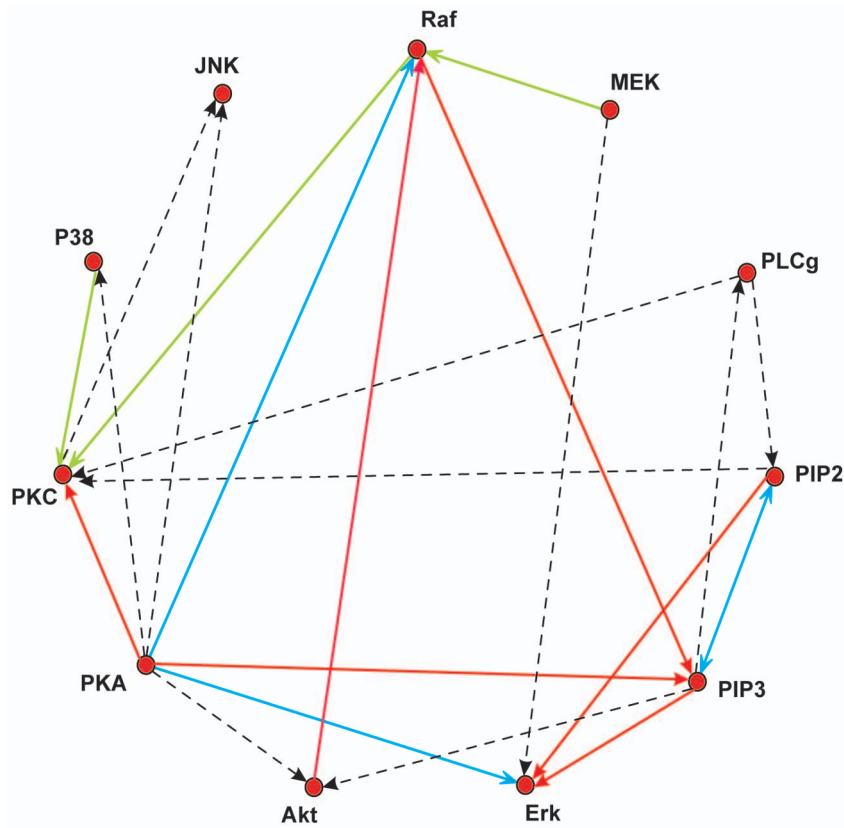


Figure 4. Signaling in CD4⁺ T-cells. LP network inference results of the flow cytometry data. The blue lines correspond to true positive edges given in the reference network from Sachs *et al.* Green edges have been predicted in the wrong direction (reversed edges) and red edges are false positives. Dashed lines are missed interactions.
doi:10.1371/journal.pone.0069220.g004

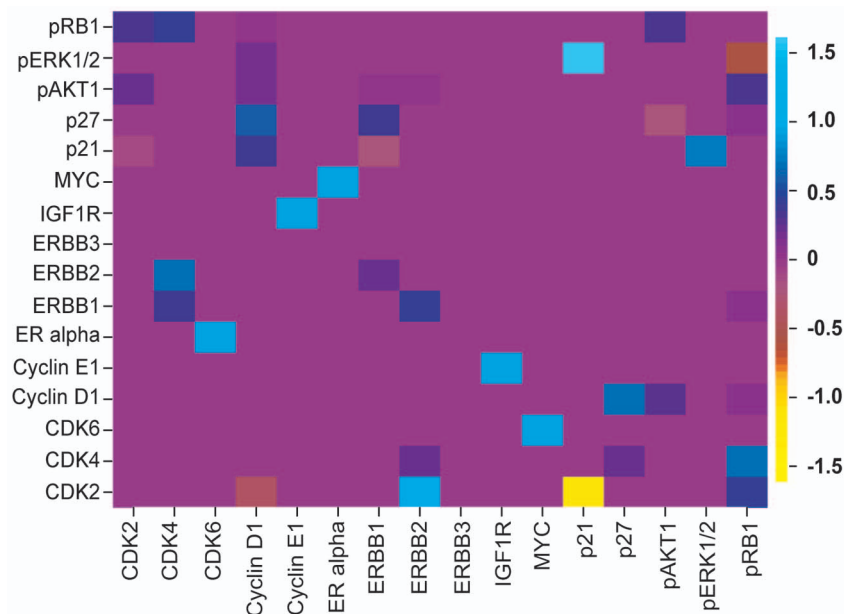


Figure 5. Reconstructed edge weights in ErbB signaling. Imageplot of median of inferred edge weights w_{ij} of the ErbB signaling data. Shown are average results from the crossvalidation runs. Parameter i refers to columns and j to rows, hence there is for example a strong inhibition of CDK2 by p21.
doi:10.1371/journal.pone.0069220.g005

and MYC [28]. The two other interactions between MYC and CDK6, and between CDK6 and ERalpha are newly predicted activations. Using the DEPNs on the same data, an indirect path was learned from MYC to CDK6: MYC \rightarrow p27 \rightarrow CDK4 \rightarrow Cyclin D1 \rightarrow CDK6 [28]. Among the remaining learned activations with lower edge weights, the activations of ERBB1 by ERBB2 and vice versa are worth mentioning, since the two kinases are known to form heterodimers [40,47].

To evaluate these results further, we used the String database (<http://string-db.org>) as reference network [48]. We use all interactions of the 16 proteins with a combined confidence score higher than 0.92. Since the interactions given in String are undirected and unsigned, we removed the edge weights of our inferred network topology as well as the signs. We then computed sensitivity, specificity, precision and accuracy for the inferred networks. In addition, we compared results against 1000 randomly generated networks, which were derived by randomly permuting the edges in the String reference network. Last but not least, we compared our results with results of the DEPN approach on the same data, as previously published [28]. We note that an analysis on the transitive closure of the reference network is not possible, since string does not contain any directionality information. Table 2 shows the complete results obtained. Both LP and DEPN achieved a specificity of 83% ($p < 0.00001$), while the LP model achieved higher sensitivity (21%, $p < 0.00001$) than DEPN (14%, $p < 0.00001$). In terms of accuracy and precision, LP outperformed DEPN (accuracy: 63%, $p = 0.13$ LP vs. 60%, $p = 0.22$ DEPN; precision: 38%, $p = 0.44$ LP vs. 28%, $p = 0.1$ DEPN).

In conclusion, we learned several already known activations and inactivations and inferred potential new interactions. The most interesting new predictions are probably those which indicate negative or positive feedback loops, since they allow it to regulate and control the G1/S cell cycle transition. Chen *et al.* showed that the ERBB response is silenced by negative feedback from active ERK [47], supporting the idea of feedback loops in ERBB signaling.

Discussion and Conclusions

With the availability of large-scale experimental datasets and easy and relatively inexpensive access to perturbation experiments, functional screens offer a direct means to elucidate cellular signaling in living cells. However, the reconstruction of signal transduction networks from perturbation data is a challenging problem that, in spite of increased attention in the last decade, still is in desperate need for novel algorithms. The problem has been shown to be NP complete even if a core topology is known, and only minimal changes to make a model consistent with experimental data are sought for [32]. Various statistical and machine learning approaches have been developed to reconstruct networks from observational data, and several address network inference from perturbation experiments. Main challenges in the field come from the complexity of the inference problem, with an exponentially growing number of possible network topologies for increasing network size. Methods such as Nested Effects Models or Deterministic Effects Propagation Networks then quickly reach computational limits when larger networks are targeted.

Our main contribution in this manuscript is the formulation of the network inference problem in terms of an information flow through a graph. While the well known max-flow/min-cut problem in graph theory searches for a maximum flow through a given network, our problem here is inverse in the sense that we know values of the flow through the network for different cuts (knockdowns), and wish to reconstruct the underlying network topology from this data. Using maximum parsimony as a guiding principle, we show how this leads to a formulation of the problem as a linear program, a class of optimization problems that has received considerable attention in combinatorial optimization. Integer linear programming is NP hard, but by making the assumption that the edge weights $w_{i,j}$ are continuous, we can formulate the network inference problem as a non-integral LP, thus making the problem solvable in polynomial time. Using a heuristic, threshold-based discretization of edge weights, we then arrive at an approximately optimal network topology. This trick allows it to solve substantially larger network inference problems, and present limitations in network inference then no longer arise from the computational complexity, but rather limited availability of perturbation data with sufficient observations of gene/protein activity levels after all knockdowns.

Data requirements for such network inference are still limiting the application of such approaches as ours on large-scale screens. For a screen with n genes, we ideally would need single knockdowns of all n genes, each with subsequent measurements of the activity levels of all n affected proteins. While large-scale screens are widely available, the second requirement – observations of activity levels of all proteins after each knockdown – is still rare and not routinely measured. Microarrays have been used to measure transcriptional activity after gene knockdown, but offer only a very indirect view about changes at the protein level [49]. To complicate matters further, cellular networks are often robustly designed [50], and single knockdowns may not be sufficient to affect cellular phenotypes in such situations due to redundancies in the cellular pathways. This can be overcome by combinatorial screens with double or multiple knockdowns, which can easily be integrated into our LP formulation in a canonical way, but further increase data requirements [51].

Albeit the approach we pursue here is using a deterministic model of signal transduction and pathway activity, we show that it can deal extremely well with noisy, stochastic data. The introduction of slack variables in the formulation of the optimization problem here is key to cope with experimental and

Table 2. Evaluation results on ErbB signaling.

	LP model	DEPN	random
TP	9	6	14.72
TN	71	71	58.72
FP	15	15	27.28
FN	33	36	27.28
SP	0.83**	0.83**	0.68
SN	0.21**	0.14**	0.35
PR	0.38	0.28	0.35
AC	0.63	0.60	0.57

Shown are comparative performance measurements for network inference on reverse phase protein array data regarding ErbB signaling in breast cancer cells. Network inference was performed using our linear programming (LP) approach, Deterministic Effects Propagation Networks (DEPN), and random guessing of a network. Results were compared with a gold standard network from the String database. TP = true positives, TN = true negatives, FP = false positives, SP = specificity, SN = sensitivity, PR = precision, AC = accuracy. Statistically significant differences are marked with ** ($p < 0.0001$) and * ($p < 0.05$), respectively.

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biological variability, and permits solution of the model even in the presence of conflicting data. The principle of maximum parsimony, i.e. minimization of the overall sum of edge weights, further helps to drive solutions to sparse networks, and makes the inference feasible even in the presence of substantial amounts of missing data. The formulation of the objective function as we use it, with minimization of the sum of absolute edge weights instead of the sum of squared weights, leads to solutions of the LP where $w_{i,j}=0$ for as many tuples (i,j) as possible, and hence sparse networks. This is known as Lasso regularization, and is a direct analogue of using a zero-mean Laplace prior in Bayesian network inference, as can immediately be seen by taking the negative logarithm of the Laplace prior. Correspondingly, the parameter λ in equation (3) has an intimate relation to the dispersion parameter of the Laplace distribution, and hence relates the expected variance of the weight parameters w to the variance of the slack variables ξ . This parameter effectively trades off variability in the experimental data and thus the slack variables to variability in the model parameters $w_{i,j}$. This implies that the value of λ should be chosen not only based on network size, but also taking into account the overall variance of the experimental measurements.

A critical issue in setting up the linear program is the choice of threshold parameters δ_i for discretization of gene activity levels. This parameter determines, for each gene i separately, from which level of activation $x_{i,k}$ a gene or protein v_i is considered active or inactive. Based on this discretization, either constraint (4) or (5) are used. Correspondingly, the choice of δ may critically affect results of the whole inference procedure. Optimally, δ should be determined experimentally from control experiments directly knocking down each gene v_i and comparing its activity with a negative control. Alternatively, statistical approaches using significance levels for false positive calls could be used to define a threshold after z-score normalization of the data, for example using the full screen as a quasi negative control [52]. A simple approach that we used in this manuscript was to calculate δ_i from the data, for example as the mean of the $x_{i,\cdot}$ for all experimental measurements of a given gene v_i .

The simulation study we performed illustrates stable performance of our approach both in light of noisy and missing data, with superior performance over the DEPN approach at least on the noisy data we simulated. On the two real experimental data sets, the LP approach shows higher sensitivity and precision, at the expense of decreased specificity in comparison to the DEPN approach. Since network inference predictions should be considered as hypotheses only and not ultimate reality, and clearly need further experimental validation to firmly establish interactions between involved proteins, it may be an advantage to rather have a higher rate of false positive predictions than too many false negatives.

Importantly, there are fundamentally different assumptions made in the underlying network models of the DEPN and LP approaches. To illustrate the difference, assume a simple network with three nodes A , B , C , and edges $A \rightarrow B$, $B \rightarrow C$, and $A \rightarrow C$ to be given. Furthermore, assume that a knockdown of node B is made. DEPNs now assume that the effect of the knockdown of node B can be observed at node C , since C is downstream of B . In terms of “activation” of a signaling molecule, this means that DEPNs assume that node C will only become activated, if all of its parents are activated, hence incoming edges are connected with *AND* in the activation function. In the given example, node C is not activated, due to the knockdown of B . In our model, in the same situation, we assume that node C will be activated after knockdown of B , due to the direct edge $A \rightarrow C$. Incoming edges to a node are thus aggregated using the *OR* function. Interestingly,

these differences in assumptions make it possible for the LP approach to infer whether or not an edge $A \rightarrow C$ exists, whereas it is impossible to make any statement about this edge using the DEPN model, since under this model, there is no difference in the observed state of node C , no matter whether the edge $A \rightarrow C$ exists or not. It is possible to change these underlying assumptions and implement DEPNs with an “OR” activation function; however, the LP model becomes a nonlinear model if “AND” interactions are assumed.

Our results not only show how the choice of method to be used depends on the research question at hand, e.g. hypothesis generation versus prediction of high confidence interactions, but also on the type and structure of the underlying data: DEPNs are suitable for small-scale networks with large amounts of available data, but rapidly deteriorate with increasing levels of noise. Furthermore, the two models assume different underlying mechanisms of signal transduction and effect propagation. The scenario where the LP approach can play its strengths are larger network inference problems with up to several dozens of genes, possibly involving missing values and higher levels of noise in the data, and ideally comprising single and combinatorial knock-downs. Both approaches require direct readouts of states of the proteins involved in the signaling network, for example from protein arrays. If only indirect observations at the transcriptomic level e.g. through microarrays or RNA sequencing technology are available, or if cellular phenotypes are observed e.g. from microscopy based screening approaches, Nested Effects Models are the method of choice in case of high-dimensional downstream readouts [21–25], or Probabilistic Boolean Threshold Networks in case of low-dimensional observations [17]. Neither DEPN in their original form nor our LP approach can directly handle time series data, which would help to reconstruct in particular feedback cycles in signaling networks. D-DEPNs are an extension of DEPNs that are specifically designed for time series experiments [29]. D-DEPNs work particularly well if long time series measurements are available for small networks. Importantly, for small time courses of large networks as tested in this manuscript, D-DEPNs are not applicable and could not be used. An obvious extension of the current LP approach therefore is to explicitly take time series measurements into account and exploit the information conveyed by the temporal evolution of a network to refine network reconstruction.

An interesting observation is the very good performance of the Bayesian network used by Sachs *et al.* on the flow cytometry study [15]. Importantly, the authors use pairwise correlations of the single-cell data to predict dependencies and causal interactions. This additional information coming from hundreds of individual cell measurements cannot be exploited by the DEPN and LP approaches, for which the relative amounts of correlated measurements need to be transformed into an observation matrix which reflects the effects of each perturbation at the average level per knockdown. Thereby, the single-cell information are lost. Integrating such single cell data into combinatorial optimization approaches to network inference is an open issue for future work.

Supporting Information

Figure S1 Evaluation on simulated data against transitively closed reference network. The figures show the area under the receiver operator characteristic (AUC ROC) and area under the precision-recall (AUC PR) curves on simulated ten-node and large-scale networks. Shown are the results for the Deterministic Effects Propagation Networks (DEPN) and random guessing of the transitively closed reference networks. (A) and (B)

show performance on data with increasing levels of noise, and (C) and (D) illustrate performance effects of increasing levels of missing data for the ten-node networks. (E) and (F) show the AUC values for the large-scale networks. (TIF)

Figure S2 Prior knowledge. The figure shows the AUC values of (A) ROC and (B) PR curves of the network inference using the LP model and random guessing on data simulated for the ten-node networks randomly selected from KEGG. The x-axis labeling denotes the percentage of interactions which are defined to be known a priori in the LP model. The LP-SF model is the model with known source and sink nodes. (TIF)

Figure S3 Evaluation with inhibitory edges. The figure shows the evaluation results of a two class ROC analysis considering only activating edges and a three class ROC analysis considering activating and inhibitory edges, as described [20]. The two class results correspond to the A ROC and B PR curves of the network inference using the LP model and random guessing on data simulated for the ten-node networks randomly selected from KEGG. For the three class evaluation we randomly set half of the edges given in each of the ten-node networks to be inhibitory. We inferred the underlying networks and computed the AUC values as described. The results are shown for the (A) ROC and (B) PR curves for the three class evaluation on the two boxplots of the right side of each figure. Note that in the three class analysis, random guessing has an AUC ROC value different from 0.5, and a PR value smaller than in the two class case. The dashed horizontal lines show the expected values for random guessing. (TIF)

Table S1 Evaluation of the DEPNs with transitively closed reference network. The table shows performance measures for the network inference on the flow cytometry data regarding signaling downstream of CD3, CD28 and LFA-1 in CD4⁺ T-cells. Network inference was performed using the Deterministic Effects Propagation Networks (DEPN) and random guessing of the transitively closed reference network (reported in

Sachs *et al.*). TP = true positives, TN = true negatives, FP = false positives, SP = specificity, SN = sensitivity, PR = precision, AC = accuracy. Statistically significant differences are marked with **($p < 0.0001$) and *($p < 0.05$), respectively. (PDF)

Table S2 Inferred edge weights flow cytometry data. Table S2 shows the average edge weights across the inferred network topologies using the LP model and the bootstrapping approach on the flow cytometry data. (PDF)

Table S3 Inferred edge weights ERBB data. Table S3 shows the median edge weights \pm the median absolute deviation (MAD) of all LOOCV-steps for the inferred network topologies, using the LP model on the ErbB signaling data. If the MAD is not given explicitly, it is equal to zero. (PDF)

Supplementary File S1 Methods and implementation details. This pdf-file provides additional information on how the ten-node and large-scale networks have been extracted from KEGG, and it gives details on the preprocessing of the flow cytometry data. (PDF)

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Author Contributions

Conceived and designed the experiments: BK LK. Performed the experiments: BK. Analyzed the data: BK. Contributed reagents/materials/analysis tools: BK. Wrote the paper: BK LK. Advised on research: LK.

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